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There is now accumulating evidence to suggest that aberrant expression of cyclin D1 and cyclin E occurs in human breast cancer and overexpression of these two oncogenes is associated with poor prognosis in primary breast cancers. To define the role of cyclin D1 and cyclin E expression as markers of therapeutic responsiveness to cancer treatment, cell lines inducibly overexpressing cyclin D1 or cyclin E under a tetracycline-controlled gene expression system were produced. Increased expression of cyclin D1 in the range of 5 - 10 fold and cyclin E in the range of 3 - 5 fold were confirmed in the stable T-47D transfectants using Western and Northern blots. Preliminary results from MTT assays suggested that overexpression of cyclin D1 might be a marker of sensitivity to antiestrogens, while overexpression of cyclin E had no effect to the sensitivity. Ongoing clonogenic survival assay has been used test responsiveness to antiestrogen ICI 182780 and a range of chemotherapeutic agents including doxorubicin, methotrexate, 5-fluorouracil, paclitaxel. The accrual of paraffin-embedded tissue blocks from patients with advanced breast cancer treated with various chemotherapeutic and endocrine regimens has been initiated to address the issue of the relationship between overexpression of cyclin D1 or cyclin E and therapeutic responsiveness to tamoxifen and anthracycline-based or CMF-type chemotherapy in the clinical setting. Experiments have also been initiated to define the effects of cyclin D1 and cyclin E overexpression on key molecular endpoints following antiestrogen treatment using the stable T-47D transfectants. This may aid in the understanding of the underlying mechanisms in determining sensitivity to cancer therapy in breast cancer.

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ANNUAL SUMMARY - Year 1

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Introduction

Cyclins belonging to the D and E families and their respective kinase partners play a pivotal role in regulating the progression of diverse cell types through G_1 phase of the cell cycle. Deregulated expression of either cyclin D1 or cyclin E can provide a growth advantage to tumor cells; their expression in the mammary gland of transgenic mice results in abnormal epithelial proliferation and adenocarcinoma, and they thus function as oncogenes. There is now accumulating evidence to suggest that aberrant expression of cyclin D1 and cyclin E occurs frequently in human breast cancer. Overexpression of these 2 genes is associated with poor prognosis in primary breast cancers. The latter could be in part due to reduced responsiveness of the tumors to systemic treatment. The aim of this project is to define the role of cyclin D1 and cyclin E as markers of therapeutic responsiveness in both preclinical and clinical models of breast cancer.

The project is designed to:
1. assess the relationship between cyclin D1/cyclin E expression and response to therapy including endocrine treatment, chemotherapy and radiation therapy in *in-vitro* studies.
2. investigate the relationship between the levels of cyclin D1 and cyclin E expression, response rate and survival in the clinical setting.
3. determine the underlying mechanisms contributing to cyclin D1 and cyclin E being markers of therapeutic responsiveness in breast cancer

Body of Report

Task 1: *In-vitro* study to determine the relationship between cyclin D1/cyclin E expression and response to cancer therapy (Months 1 - 15)

In order to address whether cyclin D1 and cyclin E are predictive markers for therapeutic responsiveness in breast cancer, cell lines inducibly overexpressing cyclin D1 or cyclin E under a tetracycline-controlled gene expression system were produced, to enable inducible but sustained overexpression of the cyclin genes. In this system, the tet-responsive transcriptional activator (tTA), comprising the wild-type Tet repressor fused to the VP16 activation domain of herpes simplex virus, regulates expression via a tet-responsive element upstream of the gene of interest. In the presence of tetracycline, the gene of interest is silent due to tetracycline binding to tTA. However, in the absence of tetracycline, the gene is activated by tTA. The tTA expression construct was successfully and stably transfected into an ER-positive human breast cancer cell line T-47D and clonal cell lines derived. These were employed to construct further clonal T-47D breast cancer cell lines overexpressing cyclin D1 and cyclin E. Twenty stable clones transfected with the cyclin D1 construct and 34 transfected with the cyclin E construct were isolated and screened for inducibility by Western analysis. Two clones overexpressing cyclin D1 (clones D1 17-1, D1 17-7) and two overexpressing cyclin E (clones long E 17-3, long E 17-14) were identified and characterised in more detail using Western and Northern analysis. These showed increased expression of cyclin D1 in the range of 5-10 fold

and increased expression of cyclin E in the range of 3 – 5 fold, comparable to that observed in breast cancer. The inducibility on withdrawing tetracycline was confirmed in transfectants overexpressing cyclin D1 or cyclin E. The expression of cyclin E was readily repressed by a low concentration (2 μg/ml) of tetracycline, but the expression of cyclin D1 was only repressed by high concentrations (10 – 15 μg/ml) of tetracycline. Growth characteristics of these clones were assessed by MTT assay and flow cytometry for S phase fraction. The growth rate and cell cycle phase distribution of clones transfected with cyclin D1 (D1 17-1, D1 17-7) were similar to the parent cell line T-47D and the vector-alone transfected control (Empty 17-2). The growth rate of the clones transfected with full-length cyclin E (long E 17-3, long E 17-14) were slower than the parent cell line T-47D, however the growth rate of these clones with or without tetracycline was similar.

Previous *in-vitro* studies from our laboratory clearly demonstrated that ectopic induction of cyclin D1 expression in ER-positive breast cancer cell lines (T-47D and MCF-7) can overcome the inhibition of cell cycle progression induced by antiestrogen (1) suggesting indirectly that cyclin D1 overexpression may confer resistance to endocrine treatment. However, another study showed that tet-inducible cyclin D1 overexpression in MCF-7 breast cancer cells does not prevent inhibition of cell growth by antiestrogens (2) . A recent clinical study from this laboratory suggested that the duration of the response to tamoxifen was significantly longer in ER-positive patients with low cyclin D1 mRNA levels than in those with high cyclin D1 (3) , implying that overexpression of cyclin D1 may confer a degree of resistance to antiestrogen therapy, although the sample size in the subgroup treated with antiestrogen in this study was small and the analyses must therefore be interpreted with caution. In the present experiments, the role of cyclin D1 expression as a marker of therapeutic responsiveness to antiestrogens was thus examined as a priority. Experiments in T-47D clonal cell lines overexpressing cyclin D1 or cyclin E to test the therapeutic responsiveness to serial 10-fold dilutions (10^{-7} to 10^{-11}) of antiestrogens 4-hydroxytamoxifen and ICI 182780 by MTT assay were performed. Interpretation of these experiments was initially complicated, given that the colour of the tetracycline interfered with the colour of the substrate sulforhodamine in the colorimetric MTT assay. These technical problems were overcome and the preliminary results suggested that overexpression of cyclin D1 might be a marker of sensitivity to antiestrogens, while overexpression of cyclin E had no effect to the sensitivity. However, this assay lacked sensitivity and an alternative assay, clonogenic cell survival was trialled. This is much more sensitive and has now been used to test responsiveness to the pure steroidal antiestrogen ICI 182780 as well as a range of chemotherapeutic agents including doxorubicin, methotrexate, 5-fluorouracil, paclitaxel. These have initially been tested over a wide (10^{-5} to 10^{-11}M) concentration range to establish dose-response in this system. A narrower range of drug concentration has now been defined for more detailed experiments with each drug. These are ongoing and results should be available in the next 2 - 3 months.

Task 2: *In-vivo* and clinical studies to determine the relationship between cyclin D1/cyclin E expression and response to cancer therapy (Months 6 - 36)

The accrual of paraffin-embedded tissue blocks from patients with advanced breast cancer from ANZ breast cancer trials 7802 and 8101 treated with various chemotherapeutic and endocrine regimens has been initiated. There will be 113 tamoxifen-treated breast cancers, 113 AC (doxorubicin and cyclophosphamide)-treated breast cancers from the ANZ breast cancer

trial 7802 and another 153 AC-treated breast cancers from the ANZ breast cancer trial 8101 to address the issue of the relationship between overexpression of cyclin D1 or cyclin E and therapeutic responsiveness to tamoxifen and anthracycline-based chemotherapy. Moreover, there will also be 155 breast cancers treated with CMF from the ANZ breast cancer trial 8101 to answer the question of whether overexpression of cyclin D1 or cyclin E is associated with therapeutic responsiveness to CMF-type chemotherapy. Once all the tissue blocks are retrieved, immunohistochemical analysis of cyclin D1 and cyclin E expression in all primary tumors will be performed. The techniques of immunohistochemistry for cyclin D1 and cyclin E have been optimised in breast tumors within the laboratory. Both clinical trials have been completed and the follow-up data are available. Data on the relationship between cyclin D1 / cyclin E expression and response rates / disease progression / survival following hormonal, anthracycline-containing or CMF-type treatment are likely to be available within the next 12 - 18 months.

The *in-vivo* experimental work has not been commenced and the establishment of T-47D clonal cell lines overexpressing cyclin D1 or cyclin E as xenografts in nude mice will await the completion of the experiments described in task 1 of this proposal.

Task 3: Study of the underlying mechanisms in determining sensitivity to cancer therapy (Months 18 - 36)

Since clonogenic assays allow time for additional experimentation in parallel, this task has already been initiated. Several molecular endpoints have been identified following acute (0 - 48 hours) treatment of MCF-7 breast cancer cells with the antiestrogen ICI 182780 in other studies from our laboratory. Inhibition of cyclin D1 gene expression with concurrent decline in cyclin D1 mRNA and protein levels is an early and critical event in antiestrogen action (4, 5) . More recently, both cyclin D1-Cdk4 (5) and cyclin E-Cdk2 activity were shown to be inhibited by antiestrogen treatment and this decline was dependent on the Cdk inhibitor p21 (6) . Moreover, ICI 182780 treatment induces accumulation of p130-E2F complexes, consistent with the arrest of MCF-7 cells in a quiescent (G_0) state (6) . Thus, experiments have been designed and initiated to define the effects of cyclin D1 and cyclin E overexpression on key molecular endpoints, including cyclin D1-Cdk4 and cyclin E-Cdk2 activity, p21 association with these complexes, p130-E2F4 complex formation.

In order to determine the susceptibility to apoptosis in cyclin D1 or cyclin E overexpressing cell lines with or without treatment, a number of different techniques in assessing apoptosis have been investigated. The presence of phosphatidylserine on the outer leaflet of apoptotic cell membranes as measured by flow cytometry following Annexin-V-Fluos and propidium iodide staining has been chosen as a suitable assay system. This technique has been optimised in our laboratory and experiments will be performed in the next 12 - 18 months.

Key Research Accomplishments

- Development of ER-positive T-47D breast cancer cell lines inducibly overexpressing cyclin D1 or cyclin E under a tetracycline-controlled gene expression system.
- Increased expression of cyclin D1 in the range of 5 - 10 fold and cyclin E in the range of 3 - 5 fold were confirmed in the stable T-47D transfectants using Western and Northern blots.
- Stable transfectants of T-47D cell lines (altogether 6 cell lines) were characterised using MTT assay and flow cytometry.
- Preliminary results from MTT assay suggested that overexpression of cyclin D1 might be a marker of sensitivity to antiestrogens, while overexpression of cyclin E had no effect to the sensitivity. This result requires confirmation by ongoing experiments using clonogenic assays.
- Experiments have been designed and initiated to define the effects of cyclin D1 and cyclin E overexpression on key molecular endpoints following antiestrogen treatment.

Reportable Outcomes

Development of cell lines:
Clonal lines of T-47D cells stably transfected with empty pTRE vector and pTRE vector containing cyclin D1 and cyclin E have been established. 2 clonal lines overexpressing cyclin D1, 2 clonal lines overexpressing cyclin E and 1 vector-alone control clonal line have been characterized.

Oral presentations:
PI was invited to speak in the Basic Sciences of Oncology Series at the NSW Cancer Council on:
- Molecular biology in breast cancer
- Endocrine therapy

Conclusions

Two clonal breast cancer cell lines overexpressing cyclin D1, 2 clonal cell lines overexpressing cyclin E and 1 vector-alone control cell line have now been well characterized. With these cell lines, we should be able to provide the most extensive data on the relationship between cyclin D1/cyclin E expression and response to cancer therapy using clonogenic survival assay within the next 12 months. Together with the clinical study in determining the expression of cyclin D1 and cyclin E in advanced breast cancer, we hope to provide a more in depth understanding of the significance of cyclin D1 or cyclin E as markers of therapeutic responsiveness in breast cancer in the next 12 to 18 months. These results can then be translated from the clinic back to the basic laboratory where various experiments designed to

define the effects of cyclin D1 and cyclin E overexpression on key molecular endpoints may provide more insight understanding of the underlying mechanism in determining sensitivity to cancer therapy in breast cancer. This may aid the management of breast cancer in the short term and may identify potential targets for modulation of drug resistance in breast cancer therapy in the long term.

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